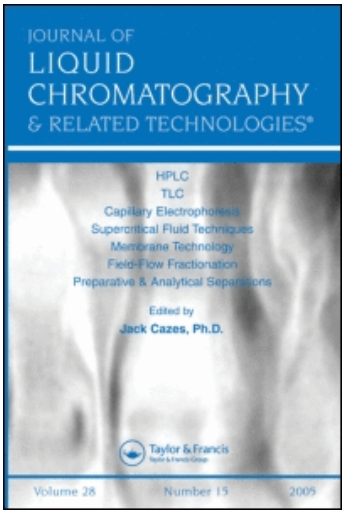

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DETERMINATION OF ALLIIN IN RAT PLASMA BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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ABSTRACT

A rapid and sensitive high performance liquid chromatography-electrospray/MS method has been developed to determine alliin in rat plasma. The method involved deproteinization of these samples with 2.5-volumes (v/w) of acetonitrile/ethanol (1:1) and then 5 μL aliquots of the supernatant were injected onto a amino-silica (NH_2) column coupled with an electrospray/MS system. The mobile phase employed isocratic elution with 5% acetonitrile for 10 min; the flow rate was 1.0 mL/min. The column effluent was analyzed by selected ion monitoring (SIM) for the negative pseudo-molecular ion of alliin $[M\text{-}H]^-$ at *m/z* 176. The limit of detection for alliin in rat plasma by this method was ca. 10 ng/mL and the accuracy for intra- and inter-assay were between 88.3% and 110.1%. The coefficients of variation of the intra- and inter-assay were generally low (below 9.2% and 11.0%, respectively) for rat plasma. No problems were encounted due to interference from endogenous substances.

INTRODUCTION

Garlic (*Allium sativum* L.) has been used worldwide as a spice and folk medicine to treat various diseases. Recent studies show that garlic extracts or compounds isolated from garlic such as sulfur containing amino acids have platelet aggregation inhibition effect,[1] protection of hepatocytes,[2,3] and cancer chemopreventive effects on gastric and colon cancer.[4,5] Among the sulfur-containing amino acids in garlic, one of the major constituents is alliin ($C_3H_5SOCH_2CH(NH_2)COOH$; S-allyl-L-cysteine sulfoxide).

A number of methods have been developed for the analysis of alliin using HPLC.[6-9] Since alliin has a poor UV-chromophore, most of these HPLC methods require a derivatization step in sample preparations with a UV- or fluorescent labeling reagent to increase sensitivity. Gas chromatographic methods also have been applied for the determination of alliin[10,11] by derivatization with bis(trimethylsilyl)fluoroacetamide or trifluoroacetic anhydride (TFAA). The drawback of these approaches is complexity of sample preparations for good sensitivity and volatility. These also may make sample preparation steps tedious and time-consuming if alliin is present in complex matrix such as plasma, urine, or biological environments.

Therefore, it was necessary to develop simple and rapid sample preparation procedures with improved analytical recovery and greater sensitivity. Electrospray is a soft ionization technique that has been shown to produce various intact ions. The method has been found useful for analysis of various molecules including peptides,[12] oligonucleotides,[13] and drug metabolites.[14]

In this paper, an HPLC-electrospray/MS method for the determination of alliin in rat plasma is described. This study is also the first application of electrospray/MS to analyze alliin, one of a sulfur-containing amino acid in garlic.

EXPERIMENTAL

Chemicals

Alliin was kindly supplied by Wakunaga Pharmaceuticals Co., Ltd. (Hiroshima, Japan). Acetonitrile (HPLC grade) and trimethylamine were obtained from Fischer Scientific Co. (Fair Lawn, NJ, USA). Water was purified using a Milli-Q system (Waters Chromatography Division, Millipore, Milford, USA). All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

HPLC-Electrospray/MS Apparatus

The HPLC-electrospray/MS system used for the analysis of alliin consisted of a Hewlett Packard (Wilmington, DE, USA) 5989B mass spectrometer, a 59987A electrospray interface, a Hitachi system (Hitachi, Japan) with a L-7100 pump, a L-7400 UV detector and a L-7250 programmable autosampler. The quadrupole temperature of the mass spectrometer was 120°C and the chromatograms were acquired in the SIM (selected ion monitoring) mode for the pseudo-molecular negative ion $[M-H]^-$ of alliin at *m/z* 176. The temperature of the drying N_2 gas was 320°C and the flow rate of nebulizing N_2 gas was 40 mL/min and maintained at 80 p.s.i. for the N_2 stream in the ion chamber.

Ion was accelerated from the electrospray ion source into the mass spectrometer and focused through hexapole and skimmers. Voltage applied for these electrical elements were optimized while constantly infusing alliin standard solution into the electrospray ion source. The entrance lens and capillary exit voltage were set 32 V and – 55V, respectively. These entrance lenses and capillary exit voltages were also optimized by monitoring the ion abundance of direct flow injection of alliin.

The HPLC separation was performed with YMC NH_2-phase column (4.6 x 250 mm) with 5 μm packing material (Wilmington, NC). The flow rate was set to 1.0 mL/min. UV detector was set to 205 nm to compare the chromatograms between HPLC-UV and HPLC-electrospray/MS. The mobile phase was water-acetonitrile (95:5), isocratic elution. The eluent was split 1:10 after the UV detector, and the smaller stream mixed with 0.2% triethylamine (methanol: water: triethylamine = 90: 9.8: 0.2, flow rate = 0.05 mL/min) solution to enhance the ionization of alliin in the MS ion chamber.

Preparation of Standard and Sample Solutions

Stock solution of alliin was prepared in water (1 mg/mL) and appropriate dilutions of the stock solution were also made with water. Standard solutions of alliin in plasma were prepared by spiking with the appropriate volume (less than 10 μL) of the diluted stock solution to plasma giving final concentration of 0.1, 0.5, 1.0, 5.0, 10.0, 20.0, and 50.0 μg/mL. Then the same procedure as the sample preparation procedure was applied to each standard solution to be used for calibration standards.

Plasma samples (100 μL) were placed in 1-mL effendorf tube and 2.5-volume of acetonitrile:ethanol (50:50) mixture was added to each plasma sample to deproteinize them. After vortex-mixing and centrifugation at 9000 g for 2 min, the supernatant was filtered through 0.22 μm membrane and then 5 μL of

aliquots of the filtrate were injected directly onto the HPLC column. After plotting the alliin standard area ratios vs. standard concentrations, the data was fit using a least square regression. The values corresponding to unknown samples were obtained by interpolation.

Accuracy, Precision, and Specificity

The accuracy and precision were investigated for both intra- and inter-assay. For intra- and inter-assay, replicate analyses (n=5) at four different drug concentrations (0.1, 1.0, 10.0, and 50.0 μg/mL) were performed. Accuracy was carried out by comparing response obtained from plasma samples added with a suitable amount of stock solutions with the corresponding to non-extracted standards in water. Precision was expressed as the percent coefficient of variation of replicate analyses (n=5) for each of four selected concentrations on a single occasion. For the specificity, samples of rat plasma from a number of subjects were tested to determine whether endogenous components would interfere with analysis.

RESULTS AND DISCUSSIONS

In order to develop an HPLC-electrospray/MS (LC-MS) method for alliin in rat plasma, flow injection analyses were performed to determine suitable electrospray parameters. Both positive and negative electrospray ionization was applied for the analysis of alliin in rat plasma. In negative electrospray ionization mode, deprotonated molecular ion $[M-H]^-$ of alliin at *m/z* 176 was observed with strong intensity; but no other adduct ions such as sodium-adducts or potassium-adducts were observed except $[M-C_3H_5-H]^-$ at *m/z* 135 (Figure 1A). In positive electrospray ionization mode, the protonated pseudo-molecular ion $[M+H]^+$ of alliin at *m/z* 178 was observed with strong intensity as well as some cluster ions (Figure 1B). These cluster ions were found at *m/z* 178, 355, 532, 709, and were identified as $[nM + H]^+$ ions (n = 2, 3, and 4), which were typical ions of alliin in positive electrospray ionization. As the sensitivity of negative pseudo-molecular ion of alliin $[M-H]^-$ at *m/z* 176 was greater than that of positive ion at *m/z* 178, the negative electrospray ionization was used for the determination of alliin in samples.

Figure 2 shows selected ion chromatograms (SICs) of drug-free rat plasma (A), drug-free rat plasma spiked with alliin (B), plasma collected at 0.5 hr after i.v. administration of 50 mg/Kg of alliin to rat observed at *m/z* 176. Under the chromatographic conditions used above, drug-free rat plasma samples were free of endogenous materials eluting with the same retention time as alliin, as were the other samples. The retention time of alliin was 4.2 min.

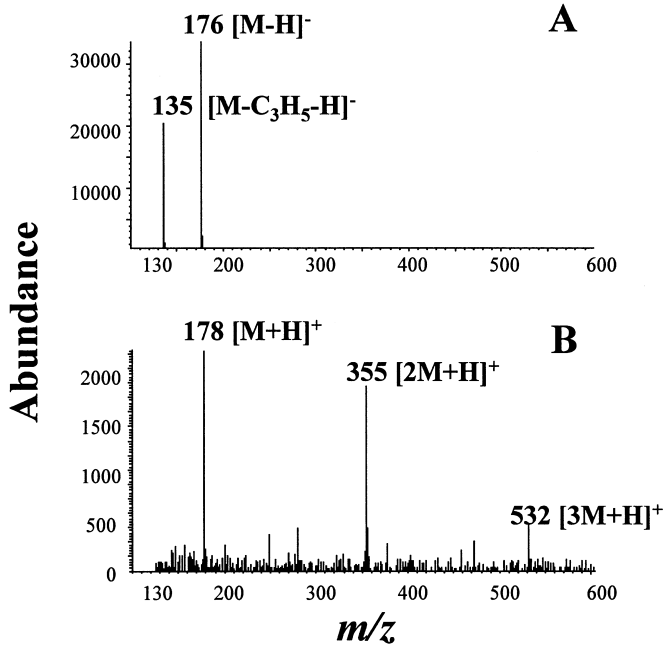

Figure 1. Electrospray-MS ionization of alliin in (A) negative ion mode and (B) positive ion mode.

For sample stability, samples obtained from plasma spiked with the different concentration of alliin were tested. Samples were stable for at least two weeks stored at 4°C. Studies carried out with frozen plasma showed that the samples were stable either at –20 or –70°C up to two months.

The calibration curve for alliin was linear (r = 0.999, regression on means, by least square analysis, of five samples at each concentration) from 0.1 to 50 μg/mL. The accuracy and precision obtained for intra- and inter-assay studies are shown in Table 1 and Table 2. The values obtained for the intra-assay and inter-assay accuracy were between 88.3% and 110.1% for alliin. The C.V. values found for the precision test for alliin were below 9.2% and 11.0% for intra- and inter-assay, respectively. To determine the detection limit of alliin, serial dilutions of alliin in mobile phase were prepared, injected and monitored at *m/z* 176. The limit of detection of HPLC-electrospray/MS for alliin was approximately 10 ng/mL based on a signal-to-noise ratio of 3 (Figure 3) and the limit of quantitation in this analysis was approximately 0.1 μg/mL.

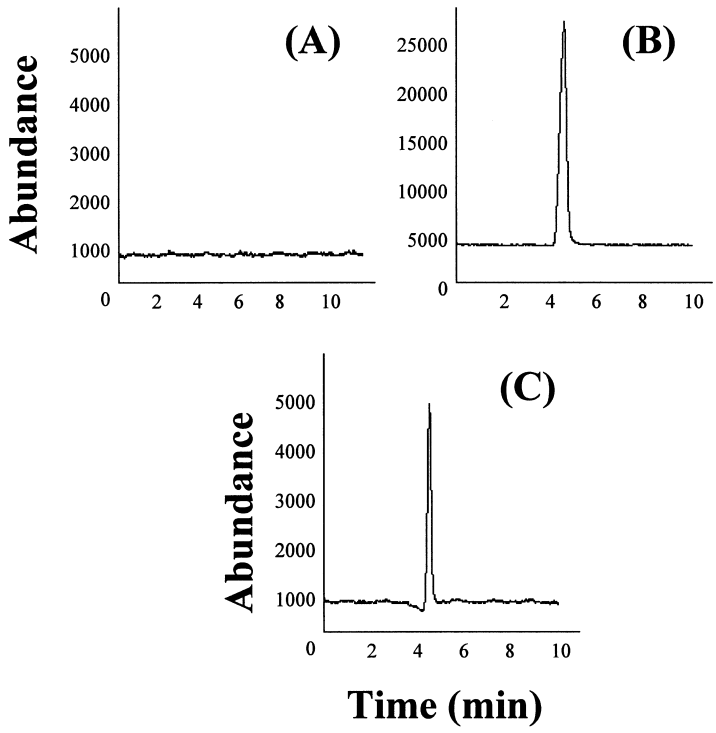

Figure 2. Selected ion chromatograms of drug-free rat plasma (A), drug-free rat plasma spiked with 5.0 μg/mL of alliin (B), rat plasma collected at 0.5 hr after i.v. administration of alliin (50 mg/Kg) (C) at *m/z* 176.

Table 1

Intra-Assay Accuracy and Precision of Alliin

Added (μg/mL)	Mean Observed Concentration (μg/mL)	Accuracy[a] (%)	C.V.[b] (%)
0.1	0.11	110	9.2
1.0	1.04	104	6.5
10.0	9.51	95.1	5.1
50.0	48.61	97.2	7.0

[a] (Mean measured concentration/Spiked concentration) x 100; mean.
[b] C.V. = (standard deviation/mean) x 100.

Table 2

Inter-Assay Accuracy and Precision of Alliin

Added (μg/mL)	Mean Observed Concentration (μg/mL)	Accuracy[a] (%)	C.V.[b] (%)
0.1	0.096	95.6	11.0
1.0	0.88	88.3	8.0
10.0	9.70	97.0	6.7
50.0	48.11	96.3	4.1

[a] (Mean measured concentration/Spiked concentration) x 100; mean.
[b] C.V. = (standard deviation/mean) x 100.

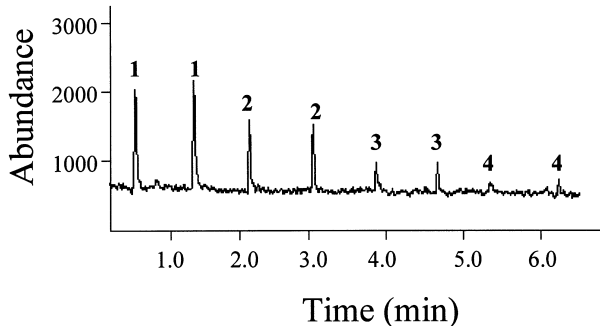

Figure 3. The limit of detection of alliin $[M-H]^-$ at *m/z* 176 using negative ion electrospray mass spectrometry with selected ion monitoring (SIM) (Signal-to-noise ratio = 3). 1: 50 ng/ml, 2: 30 ng/mL, 3: 10 ng/mL, 4: 1ng/mL.

The present method provides a sensitive and reliable technique for the determination of plasma concentrations of alliin. We surmise HPLC-electrospray/MS analysis of alliin, as described herein, should be useful for pre-clinical or clinical research application of alliin.

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